C-Terminus of Desmoyokin/AHNAK Protein is Responsible for its Translocation Between the Nucleus and Cytoplasm

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We previously demonstrated that desmoyokin gene is identical to AHNAK gene, which is downregulated in neuroblastomas. Whereas desmoyokin/AHNAK protein is distributed in the nucleus and cytoplasm in nonepithelial tissues, it is distributed in the cell membrane in epithelial tissues. It is present diffusely in the cytoplasm and nucleus of epithelial cell lines cultured in low calcium condition. Low to normal calcium shift translocates it to the cell boundary. In this study, we investigated which domain(s) of desmoyokin/AHNAK protein are responsible for its different distribution. We constructed three different eukaryotic expression plasmids, pN-DY, pM-DY, and pC-DY, which expressed N-terminus, central domain, and C-terminus of this molecule, respectively, when transfected into COS-7 cells, normal human keratinocytes, and HeLa cells. In normal calcium medium, whereas N-terminus and central domain of desmoyokin/AHNAK protein were present mainly in the cytoplasm, C-terminus was present in the nucleus, cytoplasm, and weakly in the cell membrane. In low calcium medium, C-terminus was present exclusively in the nucleus, and a part of the molecules translocated from the nucleus to the cytoplasm, 3 h after the shift to normal calcium medium or 3 h after addition of protein kinase C activator, 12-*O*-tetradecanoylphorbol-13-acetate in low calcium medium. Calcium shift showed no effects on the distribution of N-terminus and central domain. These results suggested that C-terminus, but neither N-terminus nor central domain, is responsible for the translocation of this protein into the nucleus. This study may also suggest that C-terminus play a role in the translocation to the cell membrane, although further evidence is necessary.

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Desmosomes are well-organized adhesive intercellular junctions, particularly prominent in stratified epithelia. Structural components of desmosomes consist of transmembrane glycoproteins, desmogleins and desmocollins, and nonglycosylated plaque proteins including desmoplakin and plakoglobin (Parrish *et al*, 1990; Kowalczyk *et al*, 1996; Palka and Green, 1997). Desmoyokin, a 680 kDa phosphoprotein, was also first isolated from desmosomes of bovine muzzle epidermis (Hieda *et al*, 1989). In contrast with desmoplakin, however, which locates at the center of the disk-shaped desmosomal plaques, desmoyokin was demonstrated to localize in the periphery of desmosomal plaques (Hieda *et al*, 1989). Therefore, desmoyokin was originally thought to be a stabilizer in forming and maintaining desmosomes and to be a potential adhesive molecule between stratified epithelial cells. Later, it has been reported that desmoyokin localizes substantially in the nondesmosomal areas of the cell membrane of human keratinocytes with slight accumulation around desmosome (Masunaga *et al*, 1995). Therefore, the true role of desmoyokin is obscure at present.

In the previous study, we obtained a cDNA clone DY6 that encodes a partial sequence for desmoyokin by screening a mouse keratinocyte cDNA library with an anti-desmoyokin monoclonal antibody (MoAb) 33A-3D (Hashimoto *et al*, 1993). To our surprise, cDNA sequence of desmoyokin was shown to be identical to AHNAK gene, a human gene encoding a 700 kDa protein, which was downregulated in neuroblastomas (Shtivelman *et al*, 1992). The identity between AHNAK protein and desmoyokin was further confirmed by their extensive similarity in the size, immunoreactivity and identical pattern on southern blot analysis of genomic DNA (Hashimoto *et al*, 1993).

It becomes clear that desmoyokin/AHNAK protein is present in a variety of cell types (Shtivelman *et al*, 1992; Shtirelman and Bishop, 1993; Hashimoto *et al*, 1993). Whereas desmoyokin/AHNAK protein is distributed in the nucleus and cytoplasm in nonepithelial tissues, it is distributed mainly in the cell membrane in epithelial tissues. In culture system, desmoyokin/AHNAK protein resides diffusely in the cytoplasm and nucleus of squamous cell lines in low calcium condition, and low to normal calcium shift translocates it to the cell boundary (Hashimoto *et al*, 1995).

In this study, to investigate which domain(s) are responsible for the different locations, we constructed three eukaryotic expression plasmids, pN-DY, pM-DY, and pC-DY, which expressed the N-terminus, central domain, and C-terminus of the molecule, respectively, when transfected them into COS-7 cells, normal human keratinocytes, and HeLa cells. The results indicated that the C-terminal domain, but not N-terminus or central domain, of desmoyokin/AHNAK protein is responsible for its distribution in the nucleus and its translocation between the nucleus and cytoplasm.

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Abbreviation: PoAb, polyclonal antibody.

Although the results were not very conclusive, C-terminus may also be involved in the translocation to the cell membrane.

MATERIALS AND METHODS

MoAb and polyclonal antibodies (PoAb) Rabbit anti-serum against desmoyokin/AHNAK GST bacterial fusion protein, which contained the central part of mouse desmoyokin/AHNAK protein (termed anti-desmoyokin/AHNAK protein PoAb in this study) was characterized earlier (Hashimoto *et al*, 1993, 1995). The 9E10.2 MoAb directed against a fragment of human c-myc was described previously (Evans *et al*, 1985; Stappenbeck and Green, 1992, 1993).

Cell cultures COS-7 African green monkey kidney cells were cultured in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (normal calcium medium) (Stappenbeck *et al*, 1993). Normal human keratinocytes were prepared using a standard method (Tsao *et al*, 1982), and cultured with keratinocyte-SFM (GIBCO BRL no.17005, Grand Island, NY), a serum-free medium containing 0.09 mM calcium. The cultured normal human keratinocytes were passaged weekly, and the third to fifth passages of normal human keratinocytes were used for transfection in this study. HeLa cells, a human cervical carcinoma cell line, were obtained from RIKEN gene bank (RCB0007, Tokyo) and cultured in DMEM with 10% fetal bovine serum. To prepare low calcium medium, calcium-free MEM (SMEM, GIBCO BRL, Grand Island, NY) was supplemented with 10% chelated fetal bovine serum as reported earlier (Kitajima *et al*, 1987; Hashimoto, 1988). The final concentration of calcium in the low calcium medium is about 0.02 mM. For low to normal calcium shift experiment, calcium was adjusted to 1.8 mM by adding sterilized calcium chloride.

Addition of 7-c-myc tag to an eukaryotic expression vector pcDNAI/Amp To differentiate recombinant forms of desmoyokin/HNAK protein from the endogenous one, we introduced 7-c-myc sequence as a tag to the 3′-terminus of inserts in all cDNA constructs. We examined the recombinant proteins using immunofluorescence and immunoblotting by the MoAb 9E10.2. 7-c-myc fragment was amplified by polymerase chain reaction (PCR) from plasmid p293, a pBluescript SK vector with 7-c-myc sequence inserted into its polylinker (a generous gift from Dr Joe Gall) (Roth *et al*, 1991). Sense primer was 5′-ggc*gaattc*ATGGAGCAAAAGCTCATT-3′, and anti-sense primer was 5′-ggc*ctcgag*CTAATTCAAGTCCTCTTCAGA-3′. *Eco*RI and *Xho*I sites in the primers were shown by italic letters. This PCR product was then cut with *Eco*RI and *Xho*I and subcloned into an eukaryotic expression vector pcDNAI/amp (Invitrogen Corporation, Carlsbad, CA).

Construction of pN-DY, pM-DY, and pC-DY To prepare eukaryotic plasmids expressing N-terminus (pN-DY), central domain (pM-DY), and C-terminus (pC-DY) of desmoyokin/AHNAK protein, different DNA fragments corresponding to N-terminus, four complete repeating units of central domain, and C-terminus of desmoyokin/AHNAK protein were amplified by PCR. The insert of pN-DY covers the whole 251 amino acids of N-terminus of desmoyokin/AHNAK protein. The insert of pM-DY covers the 510 amino acids of four complete repeating units of central rod domain (from residue 820 to residue 1330). The insert of pC-DY corresponds to the whole C-terminal 1002 amino acids.

As a template, we used either purified phage DNA containing genomic DNA of human AHNAK gene (Shtivelman *et al*, 1992) (a generous gift from Dr Emma Shtivelman and Professor J. Michael Bishop) for amplification of the N-terminus and central domain, or KU8 cell cDNA library (KU8 cells, a well-characterized squamous carcinoma cell line derived from a penile carcinoma) (Hashimoto *et al*, 1990) for amplification of the C-terminus. To perform the PCR, three pairs of primers were synthesized: i.e., for pN-DY, sense primer: 5′-ggc*ggatcc*GCCATG-GTTCCTGGGATAAAGGTGGG-3′, anti-sense primer: 5′-ggc*gaattc*-AGGTTTCTGAATAATCATTTC-3′, for pM-DY: sense primer: 5′-ggc*ggatcc*GCCATGGGGTCTTTGCCAGATGTTGAC-3′, anti-sense primer: 5′-ggc*gaattc*GTGCGTCTGTATATTCATGC-3′, and for pC-DY: sense primer: 5′-ggc*ggatcc*GCCATGGGGCGTCTGGATTTC-GAAGGCCCTGATGCCAAAC-3′, anti-sense primer: 5′-ggc*gaat-tc*CTCTTTCTTTGTGGAAACTGACAGCTCCAC-3′. These primers were designed according to sequence data reported previously (EMBL/GenBank/DDBJ, accession number X-65157) (Shtivelman *et al*, 1992) to generate 753 bp, 1530 bp, and 3006 bp fragments of desmoyokin/AHNAK cDNA, respectively. *Bam*HI and *Eco*RI restriction sites were incorporated into primers (as indicated in italic letters) to facilitate subsequent subcloning to the 7-c-myc-tagged eukaryotic expression vector pcDNAI/Amp prepared as described above. In each sense primer, following *Eco*RI site, a Kozak consensus sequence (GCCATGG) (Kozak, 1987) for generating a ribosome binding site and an ATG start sites were added to initiate the expression in eukaryotic cells. These constructs were driven by human cytomegalovirus promoter. PCR products were cut with *Bam*HI and *Eco*RI and ligated with *Bam*HI/*Eco*RI-cut and 7-c-myc-tagged pcDNAI/Amp. These plasmids were then transfected into XL1-Blue cells to propagate on a larger scale and purified using QIAGEN plasmids Maxi kit (QIAGEN, Chatswoth, CA). All constructs were confirmed by DNA sequencing using T7 and Sp6 sequencing primers.

DNA transfection by lipofection Plasmids were transfected into COS-7, normal human keratinocytes, and HeLa cells using the lipofection method following the manufacturer's instructions with some modification. Briefly, in all types of cells, plasmids and lipofectAMINE (Life Technologies, Gaithersburg, MD) were first diluted with serum-free DMEM. Then, plasmids and lipofectAMINE were mixed and the cells were covered. Three hours later, the medium was changed to DMEM with 10% fetal bovine serum as described previously (Felgner *et al*, 1987; Coonrod and Li Horwitz, 1997). The recombinant proteins produced by pN-DY, pM-DY, and pC-DY were named N-DY, M-DY, and C-DY, respectively. To investigate the effects of calcium on the distribution of different recombinant forms of desmoyokin/AHNAK protein, plasmids and lipofectAMINE were diluted in calcium-free MEM medium, mixed, and the cells were covered. Three hours after transfection, the medium was changed to calcium-free MEM with 10% chelated fetal bovine serum (low calcium medium). Three hours or 12 h before fixation in methanol, the calcium concentration of the medium was shifted to 1.8 mM by adding sterilized calcium chloride. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is known to activate protein kinase C directly, was demonstrated to induce the translocation of desmoyokin/AHNAK protein to the cell membrane at 10 ng per ml concentration (Hashimoto *et al*, 1995). To investigate the effects of TPA on the translocation of C-DY in COS-7 cells, COS-7 cells were transfected with pC-DY in the calcium-free MEM and cultured in low calcium medium as described above. The medium was shifted to low calcium medium supplemented with 10 ng per ml TPA, 3 h before fixation in methanol.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (Sugi *et al*, 1989; Hashimoto *et al*, 1990). COS-7 cells transfected with pN-DY, pM-DY, and pC-DY in 25 cm^2 flasks were cultured for another 2 d in MEM with 10% fetal bovine serum and collected by a plastic policeman. After a short centrifugation ($500 \times g$ for 5 min), pellets were directly dissolved in 0.5 ml sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer (Laemmli, 1970) and boiled for 5 min. Then, the samples were applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After the proteins in the gels were blotted to nitrocellulose membranes, the blots were stained with anti-c-myc MoAb 9E10.2 and anti-desmoyokin/AHNAK protein PoAb as primary antibodies. Peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins were used as secondary antibodies. Color was developed by 4-chloro-1-naphthol.

Immunofluorescence To examine endogenous desmoyokin/AHNAK protein, immunofluorescence was performed using anti-desmoyokin/AHNAK protein PoAb as reported previously (Hashimoto *et al*, 1993). To examine truncated proteins, COS-7 cells, normal human keratinocytes, and HeLa cells were transfected with pN-DY, pM-DY, and pC-DY, fixed with −20°C methanol for 10 min, and then stained with MoAb 9E10.2 as the primary antibody. In some experiments, specimens were observed using confocal laser microscopy. pDP.DN (Stappenbeck *et al*, 1992) and pcDNA3.1/Myc-His/lacZ (Invitrogen Corporation) were used as controls.

RESULTS

Expression of recombinant forms of desmoyokin/AHNAK protein The entire structure and the recombinant forms of desmoyokin/AHNAK protein are shown in **Fig 1**. Previous electron microscopic study showed that it has a N-terminal domain, a central rod domain, and a C-terminal domain (Hieda *et al*, 1989). These three domains were consistent with the DNA sequence data, indicating that the N-terminus consists of 251 amino acids, the large central rod domain consists of 4300 amino acids of repeating units of 128 residues, and C-terminus consists of 1002 amino acids (Shtivelman *et al*, 1992).

Endogenous desmoyokin/AHNAK protein is present in cultured COS-7 cells, normal human keratinocytes, and HeLa cells As we reported previously, cultured DJM-1 cells, KU-8 cells, PAM cells and MDCK cells express desmoyokin/AHNAK protein in both low and normal calcium condition (Hashimoto *et al*, 1993, 1995). Other epithelial cells used in this study, i.e., COS-7 cells, normal human keratinocytes, and HeLa cells, also expressed desmoyokin/AHNAK protein in both low and high calcium media (**Fig 2**, and data not shown). In low calcium media, the endogenous full-length desmoyokin/AHNAK protein is present in the cytoplasm and weakly in the nucleus of these cells (**Fig 2*a***, ***b***, and ***d***, ***e***). In contrast, 3 h after low to normal calcium shift, a clear cell membrane staining was seen in all the cell lines (**Fig 2*c***, ***f***).

Immunoblot analysis confirmed expression of recombinant forms of desmoyokin/AHNAK protein Two days after transfection, COS-7 cells transfected with all the three constructs expressed recombinant proteins as detected by immunoblotting with the anti-c-myc antibody MoAb (**Fig 3**, *left panel*). The molecular weights of N-DY (about 40 kDa) and C-DY (about 170 kDa) were of expected sizes. M-DY showed doublet protein bands with the upper band of about 98 kDa and the lower band of about 93 kDa. Because the expected molecular weight of M-DY is 98 kDa, the lower band was considered to be degradation product of the intact M-DY. When stained with anti-desmoyokin/AHNAK protein PoAb, only M-DY was detected (**Fig 3**, *right panel*), which showed the same sizes as those recognized by anti-c-myc MoAb 9E.10.2. This is reasonable, because this PoAb was generated by immunizing a recombinant protein of the central part of the desmoyokin/AHNAK protein.

In the high molecular weight area, there were weak smear-like bands with the top band about 680 kDa, as indicated by an arrow in **Fig 3** (*right panel*). These bands accounted for the endogenous desmoyokin/AHNAK protein, because these bands could also be seen in the blot of the untransfected COS-7 cell lysate.

C-terminus of desmoyokin/AHNAK protein located in both the nucleus and cytoplasm, and weakly in the cell membrane Cultured COS-7 cells, normal human keratinocytes, and HeLa cells were transfected with the three constructs in DMEM with 10% fetal bovine serum (normal calcium medium), although normal human keratinocytes passaged in keratinocyte-SFM medium. In all the cell lines, N-DY (**Fig 4*a–c***) and M-DY (**Fig 4*d–f***) mainly located in the cytoplasm, particularly around the nucleus. In contrast, C-DY located in both nucleus and cytoplasm (**Fig 4*g–i*** and ***j***, ***l***). The cytoplasmic staining was stronger in the perinuclear area. Weak cell membrane staining was seen at the attached area where cells formed close colonies (**Fig 4*j–l***). Most normal human keratinocytes transfected with pC-DY did not show clear cell membrane staining (**Fig 4*h***). A few normal human keratinocytes showed cell membrane staining (**Fig 4*k***), however, although the nuclear staining is not very clear in this particular figure. In confocal laser microscopy for C-DY expressed cells, much stronger granular staining in the perinuclear areas was discernible (**Fig 4*g***). Patchy cell membrane staining was also seen (**Fig 4*g***).

To exclude the possibility that the nuclear staining of C-DY may be due to endogenous c-myc protein or nonspecifically expressed c-myc protein by the c-myc sequence tagged in the vector, we

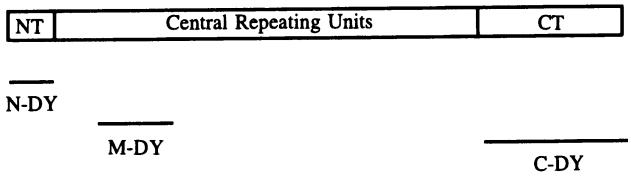

Figure 1. Schematic representation of wild and recombinant forms of desmoyokin/AHNAK protein. The wild form of desmoyokin/AHNAK protein consists of N-terminus (NT), central repeating units, and C-terminus (CT). Recombinant protein N-DY contains the whole 251 amino acids N-terminus of wild desmoyokin/AHNAK protein. M-DY consists of four complete repeating units of central domain with 510 amino acids. C-DY consists of the whole 1002 amino acids C-terminus.

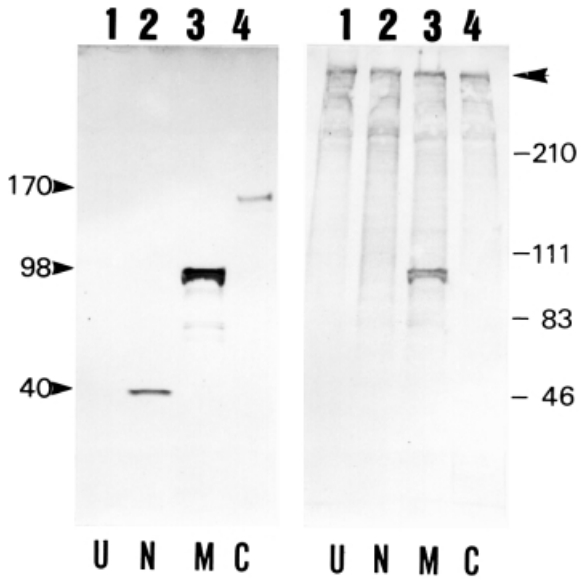

Figure 3. Immunoblot analysis confirmed that each recombinant form of desmoyokin/AHNAK protein was correctly expressed in COS-7 cells. Left panel represents blots stained with anti-c-myc MoAb 9E.10.2, and right panel represents blots stained with anti-desmoyokin/AHNAK protein PoAb. In each panel, *lane 1* (U) was blotted with untransfected COS-7 cell lysate, *lane 2* (N) was blotted with pN-DY transfected COS-7 cell lysate, *lane 3* (M) was blotted with pM-DY transfected COS-7 cell lysate; and *lane 4* (C) was blotted with pC-DY transfected COS-7 cell lysates.

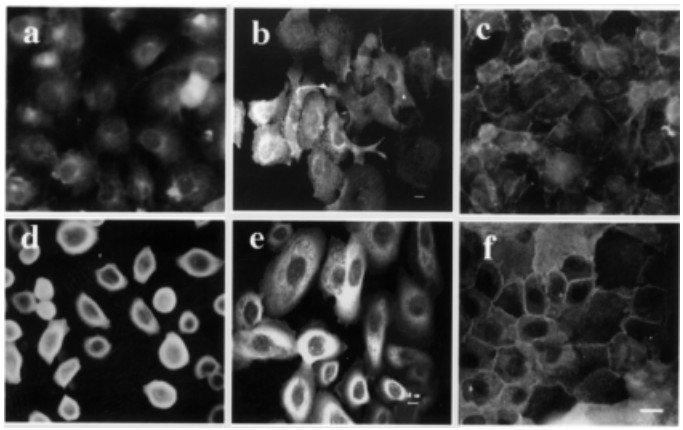

Figure 2. Endogenous desmoyokin/AHNAK protein was expressed in COS-7 cells and normal human keratinocytes in low and normal calcium media In low calcium medium (0.02 mM), endogenous full-length desmoyokin/AHNAK protein is present in the cytoplasm and weakly in the nucleus in cultured COS-7 cells (*a*, *b*) and normal human keratinocytes (*d*, *e*). (*b*, *e*) Taken by confocal laser microscopy for COS-7 cells and normal human keratinocytes, respectively, cultured in low calcium. Three hours after low to normal calcium shift, clear cell membrane staining was seen in both the cell lines (*c*, *f*). The cell membrane staining was clearly seen in the attached areas. *Scale bar*: 3 μm.

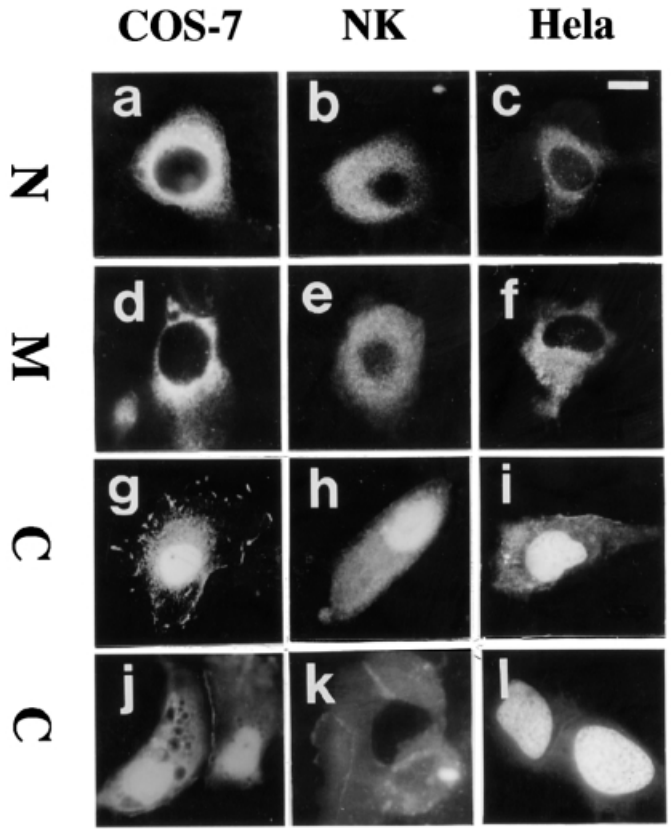

Figure 4. C-DY was seen strongly in the nucleus and weakly in the cell membrane, whereas N-DY and M-DY localized only in the cytoplasm in COS-7 cells, normal human keratinocytes, and HeLa cells cultured in normal calcium condition. COS-7 cells (*a*, *d*, *g*, *j*), normal human keratinocytes (*b*, *e*, *h*, *k*), and HeLa cells (*c*, *f*, *i*, *l*) were transfected with pN-DY (*a–c*), pM-DY (*d–f*), and pC-DY (*g–l*) in normal calcium condition. Anti-c-myc MoAb staining showed that N-DY (*a–c*) and M-DY (*d–f*) were mainly present in the cytoplasm, whereas C-DY (*g–l*) was seen strongly in the nucleus and the cytoplasm. Weak cell membrane staining was seen in the closely attached areas of pC-DY transfected cells (*j–l*). Most normal human keratinocytes transfected with pC-DY did not show clear cell membrane staining (*h*). A few normal human keratinocytes, however, showed relatively strong cell membrane staining, although the nuclear staining was not clear in this particular figure (*k*). Confocal laser microscopy showed much stronger perinuclear staining and patchy cell membrane staining in COS-7 cells transfected with pC-DY (*g*). *Scale bar*: 3 μm.

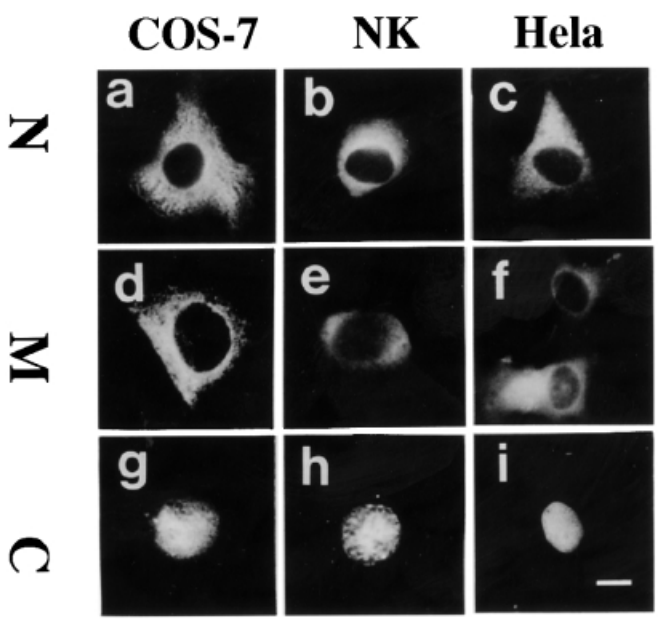

Figure 5. C-DY was confirmed to be responsible to the localization in the nucleus in COS-7 cells, natural killer cells, and HeLa cells cultured in low calcium condition. COS-7 cells (*a*, *d*, *g*), natural killer cells (*b*, *e*, *h*), and HeLa cells (*c*, *f*, *i*) were transfected with pN-DY (*a–c*), pM-DY (*d–f*), and pC-DY (*g–i*) in low calcium condition. Anti-c-myc MoAb staining showed that N-DY (*a–c*) and M-DY (*d–f*) were present in the cytoplasm, which was the same as in the normal calcium condition. In contrast, the majority of pC-DY expressing cells (*g*, *h*, *i*) showed only nuclear staining. Only *b* is a figure taken by confocal laser microscopy. *Scale bar*: 3 μm.

compared the staining pattern of C-DY expressing cells to those of cells transfected with pN-DY, pM-DY, and pDP.ΔDN, as well as pcDNA3.1/Myc-His/lacZ. All of pN-DY, pM-DY, and pDP.ΔDN contained the same 7-c-myc sequence and were expressed in the same vector. pcDNA3.1/Myc-His/lacZ was a commercially available control vector with one c-myc sequence. None of the COS-7 cells transfected with these plasmids showed an evident nuclear staining (data not shown), confirming that C-DY is responsible for the clear nuclear staining.

In order to obtain large colonies of C-DY expressed cells, we tried to obtain permanent cell lines by inserting C-terminal cDNA into vector pcDNA3.1Myc-His (Invitrogen Corporation) and transfecting this construct into HeLa cells. This construct was confirmed to express C-DY in COS-7 cells and HeLa cells by transient transfection. Although about 30 independent G418-resistant cell lines were obtained, however, these cell lines never expressed C-DY by assessed with immunofluorescence.

C-DY located predominantly in the nucleus in low calcium condition, and a part of C-DY was translocated to the cytoplasm by calcium shift and addition of TPA When all the COS-7 cells, normal human keratinocytes, and HeLa cells were transfected with pC-DY in low calcium condition, approximately 70% of C-DY expressing cells showed only nuclear staining (**Fig 5*g–i***). Other positive cells showed a strong nuclear staining with a very weak cytoplasmic staining. Three hours after shift to normal calcium medium, however, approximately 80% of positive cells showed both the nuclear and cytoplasmic staining (data not shown). Twelve hours after the calcium shift, almost all positive cells showed the dual staining, the same as that of cells directly transfected in normal calcium medium (data not shown). Distribution of N-DY and M-DY in COS-7 cells (**Fig 5*a***, ***d***), normal human keratinocytes (**Fig 5*b***, ***e***), and HeLa cells (**Fig 5*c***, ***f***) cultured in low calcium media showed no difference in staining pattern from those directly transfected in normal calcium condition.

The translocation of C-DY from the nucleus to the cytoplasm was also seen when TPA, a protein kinase C activator, was added into the medium. Three hours after 10 ng per ml TPA was added into low calcium medium, about half of the C-DY expressing cells showed both nuclear staining and cytoplasm staining (**Fig 6*b***), which was in contrast to the exclusive nuclear staining seen in low calcium medium (**Fig 6*a***).

DISCUSSION

In this study, we demonstrated that the C-terminus of desmoyokin/AHNAK protein might play an important role in its translocation between the nucleus and cytoplasm. In the normal calcium condition, C-DY was located in both the nucleus and cytoplasm. In low calcium medium, the majority of C-DY positive cells showed only nuclear staining. Low to normal calcium shift induced translocation of a part of C-DY from the nucleus to the cytoplasm. These distributions of C-DY were similar to those of the endogenous desmoyokin/AHNAK protein, as shown previously (Hashimoto *et al*, 1995). Therefore, the translocation of C-DY is considered to follow the way of the full-length of the molecule. The nuclear localization of C-terminus of desmoyokin/AHNAK protein is consistent with the finding in the previous

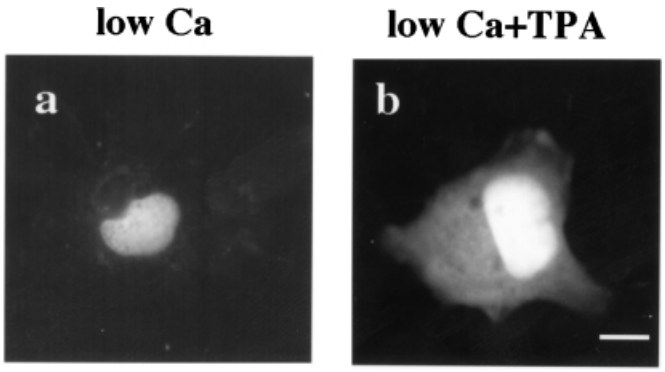

Figure 6. A part of C-DY was translocated from the nucleus to the cytoplasm by addition of TPA into low calcium medium C-DY was located mainly in the nucleus in COS-7 cells in low calcium medium (*a*). Three hours after addition of 10 ng per ml TPA in the medium, a part of C-DY was translocated to the cytoplasm (*b*). *Scale bar*: 3 μm.

amino acid sequence analysis. There are three copies of the sequence Lys–Ser–Pro–Lys, which is identical to the cdc2 kinase phosphorylation site in the histone protein, and several stretches of sequences with the high density of positively charged residues indicating possible nuclear localization signals (Silver, 1991; Shtivelman *et al*, 1992).

The N-terminus and central domain seem to be unrelated to its nuclear localization. The central domain consists of repeating units rich in glycine and proline, showing some homology with collagen and elastin families. Therefore, the central domain is likely to provide a structural function (Shtivelman *et al*, 1992). In low calcium, N-DY and M-DY located in the cytoplasm, particularly around the nucleus, but low to normal calcium shifts did not change the localization. This may indicate that desmoyokin/AHNAK protein is not closely related with desmoplakin or keratin intermediate filaments, because by low to normal calcium shift, desmoplakin translocates into the cell membrane (Sheu *et al*, 1989; Hashimoto *et al*, 1995) and keratin intermediate filaments are induced to form a radiation arrangement in the cytoplasm (Kitajima *et al*, 1988). C-DY also showed apparently different localization from those of desmoplakin and keratin intermediate filaments.

In the human and bovine epidermis, desmoyokin/AHNAK protein was clearly demonstrated in the cell membrane (Hieda *et al*, 1989; Hashimoto *et al*, 1993). Therefore, another purpose of this study was to determine which domain is responsible for its translocation to the cell membrane in epithelial cells. Only a very weak cell membrane staining could be seen in C-DY expressing cells of the attached areas of all the three cell types. We speculated that desmoyokin/AHNAK protein is accumulated to the cell membrane only in cultured cells, which attached closely to each other. The transfected cells in this study hardly formed large and closely associated colonies. In order to overcome this problem, we attempted to prepare permanent cell lines expressing C-DY. We could not, however, obtain any permanent cell lines. One possible explanation is that the C-DY overexpressed cells died out due to dominant negative phenomenon, and the cells, which obtained the G418-resistant gene fragment from the construct but excluded pC-DY insert DNA could survive. Secondly, expressed recombinant C-DY may compete with endogenous desmoyokin/AHNAK protein function and kill the cells. Nevertheless, some cell membrane staining of C-DY was reproducibly seen in all the three cell lines and this staining pattern was never seen for either N-DY or M-DY. These results strongly suggested that the C-terminal domain of desmoyokin/AHNAk protein also plays a role in the translocation to the cell membrane. Further studies are necessary to confirm this speculation.

The significance of the translocation of desmoyokin/AHNAK protein is unknown. Initially, desmoyokin was isolated as a possible structural component of desmosomes. Later studies including this study, however, showed no association of this protein with desmosomes.

Desmoyokin/AHNAK protein distributes throughout the cell membrane, cytoplasm, and nucleus, translocates by calcium shift, and is regulated by protein kinase C (Hashimoto *et al*, 1995). These findings strongly suggested that desmoyokin/AHNAK protein plays a role in signal transduction. Plakophilins have been reported to show the similar dual localization in the desmosomes and nucleus, and such a dual localization was speculated to confer a function of signal transduction on the Armadillo repeating units (Hatzfeld *et al*, 1994; Schmidt *et al*, 1997). Although desmoyokin/AHNAK protein has repeating units in the central domain, however, it has no similarity with Armadillo repeat. This study indicated that the translocation of desmoyokin /AHNAK protein is likely to be regulated by its C-terminus, which may contain a novel type of nuclear localization signal.

Lastly, it is unknown why desmoyokin/AHNAK protein shows different localization between epithelial cells and nonepithelial cells, such as 3T3 cells, melanoma cells, or neuroblastoma cells. The localization of different domains of desmoyokin/AHNAK protein should be investigated in nonepithelial cells in the future.

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